Journal of Hazardous Materials 185 (2011) 591–597

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Biological effects and photodegradation by TiO_2 of terpenes present in industrial wastewater

Irene Catanzaro[a], Giuseppe Avellone[b], Giuseppe Marcì[c], Marghereth Saverini[a], Lea Scalici[b], Giulia Sciandrello[a], Leonardo Palmisano[c,*]

[a] Dipartimento di Biologia Cellulare e dello Sviluppo "Alberto Monroy", Università di Palermo, Viale delle Scienze, 90128 Palermo, Italy
[b] Dipartimento di Chimica e Tecnologie Farmaceutiche, Università di Palermo, Via Archirafi 32, 90123 Palermo, Italy
[c] Dipartimento di Ingegneria Chimica dei Processi e dei Materiali, "Schiavello-Grillone Photocatalysis Group", Università di Palermo, Viale delle Scienze, 90128 Palermo, Italy

ARTICLE INFO

Article history:
Received 5 July 2010
Received in revised form
15 September 2010
Accepted 16 September 2010
Available online 16 October 2010

Keywords:
Terpenes clonogenic assay
Terpenes mutation assay
TiO_2 photocatalysis

ABSTRACT

The aim of this work was to study the biological effects of four monoterpenes, i.e. α-pinene, β-pinene, 3-carene and D-limonene present in the wastewater of a citrus transformation factory. The study was carried out by exposing V79 Chinese hamster cells to single terpene or to the mixture of four terpenes at concentrations corresponding to those in the wastewater evaluated by head space solid phase micro extraction and gas chromatography (HS-SPME-GC) analyses. Treatments with single or combined terpenes similarly affected cell vitality, but only the combined treatments induced the 6-thioguanine resistant mutants. Moreover the photocatalytic degradation of the four terpenes was successfully achieved with the photocatalyst TiO_2 Degussa P25 in both the actual effluent and in synthetic solutions.

1. Introduction

Terpenes are a family of hydrocarbons present in essential oils of a great variety of plants to which they confer a particular fragrance. They carry out important functions inside the plants related to their growth and moreover they work as a communication language with the environment. In fact a lot of both volatile and non-volatile terpenes are involved in the attraction of pollinators and in defence against predator insects, herbivores and microbes [1–3]. They are secondary metabolites produced from photosynthetic carbon through the mevalonate pathway. The main enzymes involved in this process are terpene synthases which are organized in a large gene families; it is know that the expression of these genes is regulated not only by temporal or spatial factors but also by some biotic and abiotic stimuli. This high regulation determines the production of different types of terpenes distributed in different parts of the plant such as flowers, fruits, leafs and roots [4–6]. In the past, essential oils have been used as natural remedy [7]; today they are also added in fruit juices, soft drinks, ice cream as flavouring agents; moreover, they are present in house detergents, cleaning products, soaps, perfumes and cosmetics. Since essential oils and therefore terpenes, are widely diffused in the environment and particularly in water, because of human use, and in air, because of natural emission, it is also important to establish if these molecules are dangerous for environment and human health. Only few conflicting studies exist [8–10] on the biological effects of essentials oils and terpenes that are ubiquitous in the environment [11]. It is worth noting that essential oils have a broad spectrum of bioactivity because of the presence of several active components working in various ways [12]. Menthol, for example, has been used since many years in medication for throat irritation. Recently this terpene has been also used as additive in cigarettes and it has been hypothesized that it is responsible for increasing the nicotine dependence and for causing respiratory depression [13]. Heterogeneous photocatalysis is one of the advanced processes of oxidation more developed in the last years [14,15]; this process uses semiconductor oxides irradiated with UV or near-UV light at ambient temperature and pressure and in the presence of oxygen. The fundamental mechanism of photocatalysis consists in the generation of electron–hole pairs, which determine the occurrence of redox reactions of species adsorbed on the photocatalyst surface. This method has been successfully used for wastewater treatment and it is suitable to perform the complete degradation of organic and inorganic pollutants, the reduction of several metal ions, the inactivation of many aerobic bacteria, etc. Titanium dioxide is extensively used as photocatalyst due to its high chemical stability, optical and electronic properties [16], low cost, and absence of toxicity. The aim of the present study was to evaluate biological effects in cultured mammalian cells of four terpenes, i.e. α-pinene, β-pinene, D-limonene and 3-carene present

* Corresponding author. Tel.: +39 09123863746.
E-mail address: palmisano@dicpm.unipa.it (L. Palmisano).

0304-3894/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jhazmat.2010.09.059

in actual aqueous effluents derived from a citrus fruit transformation factory that utilizes biological treatments to purify wastewater. Little information is known on their genotoxicity and their possible photocatalytic degradation. An analytical investigation by HS-SPME-GC was carried out and the possibility to photodegrade these molecules by using polycrystalline TiO_2 irradiated with UV light was investigated. Both synthetic solutions of the four terpenes and some actual samples containing dissolved terpenes were subjected to photocatalytic tests. The terpenes listed above are some of the most important terpenes present in a citrus essential oil and were considered since were those found in highest concentrations in the actual industrial effluents.

2. Experimental

2.1. Chemicals

Terpenes were purchased from Sigma–Aldrich. For the biological study fresh solutions were prepared by dissolving each of them in a complete medium supplemented with 0.5% DMSO (Sigma), while methanolic solutions were prepared for the analytical study. The photocatalytic experiments were carried out by using aqueous solutions of the substrates.

2.2. Wastewater samplings

Samples were collected in a citrus fruit transformation factory that utilizes biological treatments to purify wastewater. The types of aqueous effluents studied were: washing water (A), untreated (B) and treated (C) wastewater. Three samples were collected at each sampling location named A, B, and C during the period of activity of the factory between 2008 and 2009 (see Fig. 1).

2.3. HS-SPME-GC analyses

The combination of HS-SPME-GC/FID offers high instrumental sensitivity allowing for the simultaneous identification and quantification of each terpene both in the industrial and synthetic samples. 110 ml of each type of aqueous actual effluent were poured into a 125 ml amber bottle. The bottle was immediately plugged with septa in polytetrafluoroethylene (PTFE)/silicon and maintained at 4 °C. Just before the analyses the bottle was equilibrated at 40 °C for 30 min to achieve the partition equilibration of volatile compounds between the sample and the headspace. The use of HS-SPME allowed both the extraction of VOCs from various kinds of matrices and their concentration before the analyses [17,18]. The determination of the quantity of VOCs in water implied the use of equilibrium partitioning coefficients at the air–water interface. In fact, these coefficients depend on pressure, adsorption time and temperature [19]. At constant temperatures each volatile compound contained in the sample was distributed among fibre–air–water phases. The quantity of terpenes contained in each phase depended exclusively on their original concentration in the water sample when the water and air volumes (head space) and the adsorption temperature were set. The choice of the fibre to be used throughout this study was done by testing preliminary a HS-SPME device and the fused silica fibres coated with PDMS having 100 μm thickness, or with a Carboxen/polydimethylsiloxane (CAR/PDMS) having 85 μm thickness, or with a divinylbenzene/Carboxen/polydimethylsiloxane (DVB/Carboxen/PDMS) having 50/30 μm thickness (Supelco). In all cases the fibres were conditioned following the indications of the producer. The monophase PDMS fibre/HS-SPME was the best one according to the Fij criterion function introduced by Zuba et al. [20] and modified by Hamm et al. [21]. Adsorption and desorption times were optimized by performing the tests at different times, thus obtaining a compromise between the speed of the analytical procedure and the recovery of the sample. The adsorption time was fixed at 10 min and the desorption of the fibre was allowed to occur in the injector at 250 °C for 2 min in splitless mode. A complete analysis run of 52 min, including extraction time, is necessary to quantitatively recognize all the compounds. For each sample three SPME extractions and desorptions were realized. All the analyses of the synthetic solutions and the linear calibration plots (R between 0.993 and 0.999) were performed using stock solutions obtained by diluting the four standards methanolic terpenes solutions (200 ppm) in ultrapure water to concentrations ranging between 5 and 500 ppb (standard deviation <15%). The limit of detection (LOD) was fixed between 1 and 3 ppb, i.e. when the signal was three times higher than the noise. Conversely, the limit of quantization (LOQ) was fixed between 3 and 5 ppb, i.e. when signal was 10 times higher than the noise. Chromatographic analyses were performed on a Shimadzu GC 2010A equipped with a Supelcowax (CW) column (Supelco) 30 m long, 0.25 mm I.D., 2.5 μm thick and a FID detector. A temperature programme from 60 °C (maintained for 3 min) to 130 °C with 15 °C/min ramp rate and then to 240 °C (maintained for 1 min) was set. The linear velocity of the carrier gas (He, 99.9995%) was fixed at 25.9 cm/s. The FID temperature was set at 250 °C.

2.4. Cell culture

V79 Chinese hamster cells were cultured in D-MEM (Gibco, Invitrogen), supplemented with 5% foetal calf serum (Invitrogen), penicillin (100 U/ml) and streptomycin (100 μg/ml) and maintained at 37 °C in a 5% CO_2 humidified incubator.

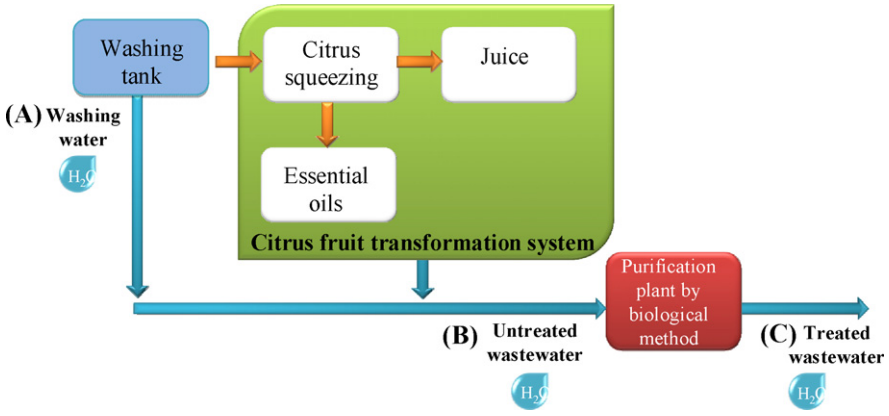

Fig. 1. Schematic representation of the citrus fruit transformation system. (A), (B), and (C) indicate the washing, the untreated and the treated wastewater samples.

2.5. Clonogenic assay

V79 hamster cells were seeded at 3×10^2/dish and incubated for 18 h. For each sample 3 dishes were prepared. Cells were exposed for 1 h to single terpenes at concentration corresponding to those found in the washing water (A) for 1 h, and to aqueous solutions containing the four terpenes at concentrations corresponding to those revealed in the washing water (A), untreated (B) or treated (C) wastewater for 1, 6, 12, 24 and 36 h at 37 °C. After the treatment, the cells were washed twice with Hanks' salt solution and left in fresh medium for 10 days. Colonies were stained with 0.1% methylene blue and counted, the relative cell survival calculated and compared to the control (V79 untreated cells). Three independent experiments were carried out for treated and unexposed cells (Control). Data are presented as mean ± SD (standard deviation). Statistical analysis was performed using the Student *t*-test.

2.6. Mutation assay

V79 hamster cells were seeded at 5×10^6 cells/dish, incubated for 18 h and then treated for 1 h at 37 °C with single terpenes at concentrations corresponding to those revealed in the washing water (A), untreated (B) and treated (C) wastewater. After treatment the cells were washed twice, collected by trypsinization and counted. The untreated culture was also washed twice and counted. Cells for each culture were respread into ten 100 mm dishes (5×10^5 cells/dish) in a medium containing 30 μg/ml 6-thioguanine (TG, Sigma) for TG^r clone selection or into three 60 mm dishes (3×10^2 cells/dish) in non-selective medium to estimate cell survival at T_0. For each culture, in addition, 10^6 cells were also subcultured in fresh medium and incubated to allow the expression of the mutation for further 72 h. At that time (T_{72}), the cultures derived from each treatment were tested for TG^r clone selection and survival. After 12 days incubation, the total number of colonies surviving in TG and those growing in non-selective medium were counted to estimate the TG^r mutant frequency calculated as the number of mutants per 10^6 survivors. The response was considered significative when the mutant frequency was at least more than twice the spontaneous frequency. Three independent experiments were carried out for treated and untreated cells. Data are presented as means ± SD (standard deviation). Statistical analysis was performed using the Student *t*-test.

2.7. Photocatalytic experiments

Aqueous synthetic solutions containing the four terpenes in concentration of 8.40 mg/l (6.16×10^{-5} M) were prepared using commercial standards. The concentration used in the photocatalytic runs was up to ca. three order of magnitude higher than those found in the washing, untreated and treated wastewater of the studied citrus fruit transformation factory. Photocatalytic degradation experiments were carried out in a hermetically sealed photoreactor (V = 280 ml) containing 100 ml of substrate aqueous solution and 0.4 g l^{-1} of polycrystalline TiO_2 Degussa P25 (ca. 80% anatase; 20% rutile). Before starting the irradiation, the reacting suspension was magnetically mixed for ca. 30 min to achieve the adsorption/desorption equilibrium of the terpenes. The artificial radiation source was a 400 W medium pressure mercury lamp (Helios Italquartz) placed 20 cm from the reacting suspension. The lamp was placed externally and irradiated the photoreactor from the above. A water filter was located between the lamp and the photoreactor to cut-off the infrared radiation and to maintain the temperature inside the reactor at approximately 300 K. The irradiance reaching the reacting suspension, measured in the wavelength range 320–390 nm with a UVX Digital radiometer, was ca. 1.5 mW cm^{-2}. In order to monitor the evolution of the terpenes concentration during the runs, samples of the gas phase (200 μl) present in the head space were withdrawn from the reactor and analyzed by using a Shimadzu GC-17A gas-chromatograph equipped with an Alltech AT-1 (30 m × 0.53 mm ID) column and a FID. Standard solutions of each terpene were prepared and calibration plots were drawn and used. The amount of CO_2 deriving from the complete oxidation of carbon present in the substrates was monitored throughout the runs using a HP6890 gas chromatograph equipped with a Carboxen column and a TCD. The experiments with the industrial wastewater were carried out in a 1.5 l Pyrex cylindrical batch photoreactor with an immersed lamp, continuously bubbling oxygen in the suspension and using an amount of TiO_2 Degussa P25 equal to 0.4 g l^{-1}. The suspensions were magnetically stirred throughout the runs and irradiated by a medium pressure mercury 500 W lamp (Helios Italquartz, Milano). The irradiance reaching the reacting suspension, measured in the wavelength range 320–390 nm with a UVX Digital radiometer, was ca. 17 mW cm^{-2}. In these cases, due to the complexity of the solution in which all the four terpenes under investigation were present along with other unknown species, only the total dissolved organic carbon (TOC) was analyzed. The analyses were performed at fixed intervals of time by using a Shimadzu 5000A instrument (error: ±1 ppm), after separation of the photocatalyst from the suspension with 0.1 μm filters (Millex, Millipore).

Table 1
Concentrations (ppb) of terpenes found in sample A, B and C derived from the citrus transformation factory (2008).

	α-Pinene	β-Pinene	3-Carene	D-limonene
A	58.12	144.21	4.02	11.09
B	85.29	525.19	103.10	181.25
C	Negligible	Negligible	Negligible	16.07

washing water (A), untreated (B) and treated (C) wastewater.

3. Results and discussion

3.1. HS-SPME-GC analyses

The concentrations of the terpenes under investigation found in 2008 in samples A, B and C, that correspond to washing water, untreated and treated wastewater, respectively, are reported in Table 1. The results indicate that all the four terpenes were present in samples A and B at different concentrations. It can be observed that the concentrations of terpenes were higher in sample B, due to the fact that in this effluent washing water and water from citrus fruit transformation were collected. Sample C, instead, showed only the presence of D-limonene, and its concentration is one order of magnitude smaller that the starting one (sample B). Representative chromatograms are reported in Fig. 2a and b.

Another sample C was withdrawn after ca. 1 year from the same plant sketched in Fig. 1 and the analytical figures are reported in Table 2. In this case D-limonene was the most abundant compound present in the treated effluents (sample C) and also the other three terpenes were present, although in small concentrations. In this case the untreated effluent B was not available, and probably the observed results can be explained by considering a different and

Table 2
Concentrations (ppb) of terpenes found in sample A and C derived from the citrus transformation factory (2009).

	α-Pinene	β-Pinene	3-Carene	D-limonene
A	271.15	1357.67	289.10	28.49
C	2.98	1.24	0.59	3.79

Washing water (A) and treated wastewater (C).

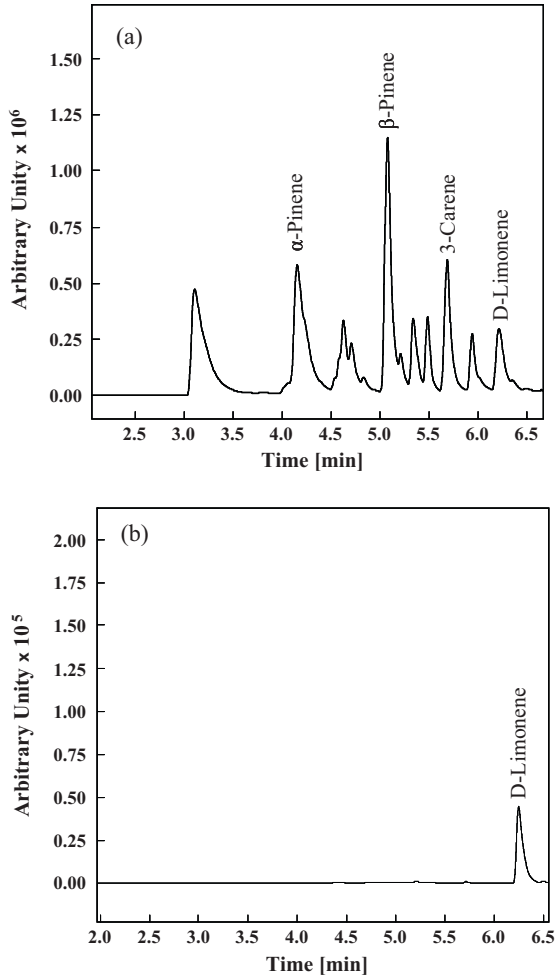

Fig. 2. (a) Chromatogram of sample B (2008) and (b) Chromatogram of sample C (2008).

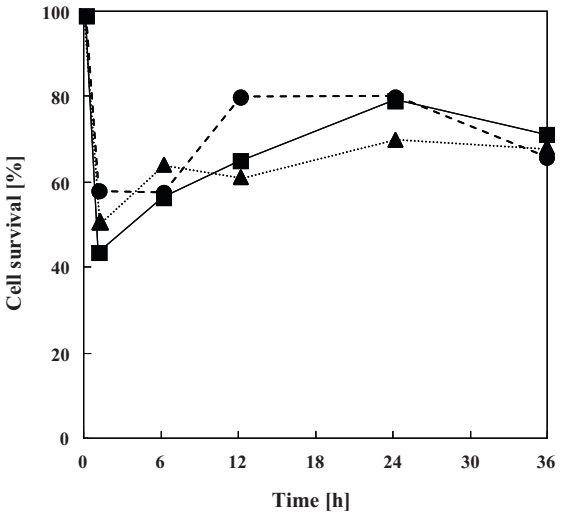

Fig. 3. Relative cell survival after exposure for different times to synthetic solution of four terpenes present in wastewater. The concentrations of terpenes correspond to the actual ones found in A (●), B (■), C (▲) samples withdrawn in 2008. The reported values are the average of measurements obtained in three independent experiments.

much higher concentration of terpenes in the effluent before the treatment.

Moreover, a further sample A corresponding to the washing water (2009) was analyzed (Table 2). It should be noted, however, that also the washing water is treated in the plant (see Fig. 1) along with the wastewater deriving from the transformation system (sample B). The concentrations of terpenes reported in Table 2 are 1–3 orders of magnitude higher with respect to the treated sample C. The analytical figures above presented indicate on the whole that the industrial biological treatment was quite effective, although residual concentrations of terpenes were still present in the treated samples. It is worth noting that both the starting and the final concentrations of terpenes can change in an actual situation, depending on the time when the sample was collected. Nevertheless the results suggested both to investigate on possible mutagenic and genotoxic effects of the four terpenes and to prove the effectiveness of an advanced oxidation technology as heterogeneous photocatalysis to achieve their complete degradation.

3.2. Clonogenic assay

To asses toxic effects of single terpene, V79 Hamster chinese cells were exposed to single terpene at concentration corresponding to those revealed in the untreated water (B). Results of clonogenic assay showed that the survival after exposure for 1 h to individual terpene decreased at about 50% (Table 3). Thereafter, cells were exposed for a longer time (up to 36 h) to aqueous solutions containing the four terpenes in concentrations corresponding to those revealed in the washing (A), untreated (B) or treated wastewater (C). The results obtained are showed in Fig. 3. The shape of the curves indicates that the toxic effects of terpenes mixture decrease by increasing the time of exposure. In fact, the cell survival was of 57.71%, 43.2% and 50.9% after 1 h of exposure to aqueous solutions A, B or C, respectively. On the contrary, for longer exposure times, the cell survival increased and a plateau was rached after 24 h. No additive or sinergistic effect on cell survival in comparison to that observed with the single compounds was detectable when the mixture was used. The lack of a time dependent increase of toxicity of the mixture could be due to the high volatility of terpenes leading to a decrease of their concentration. Based on these preliminary experiments, an exposure time of 1 h was chosen for assessing the genotoxicity of the four terpenes.

Table 3
Relative cell survival and frequency of 6-TG-resistant clones induced by exposure for 1 h to single terpene in concentrations corresponding to those present in washing water (A, 2008). The reported values are the average of measurements obtained in three independent experiments (±means standard deviation, SD).

Treatment	Relative cell survival (%)	Mutants 6TG resist./10^6 survivors (T_0)	Mutants 6TG resist./10^6 survivors (T_{72})
Untreated	100	1.4 ± 0.2	1.4 ± 0.2
α-Pinene	54.5 ± 3.7	0.4 ± 0.3	0.8 ± 0.1
β-Pinene	58.8 ± 6.2	0.8 ± 0.6	0.2 ± 0.1
D-limonene	51.5 ± 8.9	1.9 ± 1.3	1.1 ± 0.2
3-Carene	54.7 ± 8.8	0.8 ± 0.5	0.2 ± 0.03

Table 4
Relative cell survival and frequency of 6-TG-resistant clones induced by exposure for 1 h to the mixture of the four terpenes, in concentrations corresponding to those present in actual wastewater withdrawn in 2008. The reported values are the average of measurements obtained in three independent experiments (±means standard deviation, SD).

Treatment	Relative cell survival (%)	Mutants 6TG resist./10^6 survivors (T_0)	Increase versus untreated T_0	Mutants 6TG resist./10^6 survivors (T_{72})	Increase versus untreated T_{72}
Untreated	100	1.4 ± 0.2	1	1.4 ± 0.2	1
A	57.7 ± 2.7	4.1 ± 2.9	2.9	7.8 ± 1.8	5.5*
B	43.2 ± 6.8	3.9 ± 0.5	2.8	8.4 ± 1.5	6.1*
C	50.9 ± 4.9	2.1 ± 1.8	1.5	3.9 ± 0.7	2.8

* $p < 0.025$, significant increase.

3.3. Mutation assay

In order to evaluate the potential of the four terpenes to induce gene mutation, we utilized the HGPRT test. The *hgprt* gene is located on the X-chromosome and codes for the hypoxanthine–guanine phospho-ribosyl- transferase enzyme (HGPRT). The HGPRT catalyses the conversion of guanine and hypoxanthine, but also of 6-thioguanine (TG), the analogue toxic purine, to the corresponding nucleoside 5′-mono-phosphates. Mutant cells with non-functional HGPRT enzyme do not incorporate TG and thus they are able to survive in selective medium containing this analogue toxic compound. The mutagenic effect of each terpene found in washing water, was evaluated in V79 cells just after exposure (T_0) and in cells growing for 72 h (T_{72}) before plating them into a medium containing TG. Results obtained showed that the exposure at a single terpene did not virtually increase the spontaneous mutation frequency of untreated cells (1.4/10^6 cells) (Table 3). On the contrary, the concurrent exposure to four terpenes affected the frequency of mutats at the *hgprt* locus. A significant increase of the frequency of 6-TG resistant clones at T_{72} was detected in cells treated with concentrations corresponding to those determined in the washing water (A) and in the water collected before the biological treatment (B), while a slight increase in cells exposed to terpenes concentrations corresponding to wastewater biologically treated (C) was observed (Table 4). The results suggest that chemical and biological interactions may occur in the mixture of the four terpenes. Indeed, a synergistic effect can be noticed for the mutation frequency, as the effect of the terpenes mixture exceed that obtained as sum of the net effects of the four components. However, it cannot be ruled out the possibility that this result can simply be the consequence of a sublinear dose–response relationship for the single substances. The valutation of the *in vitro* results should be considered with caution, although the observed increase of DNA damage over background level provides the first indication of the potential deleterious effects of terpenes on environmental health.

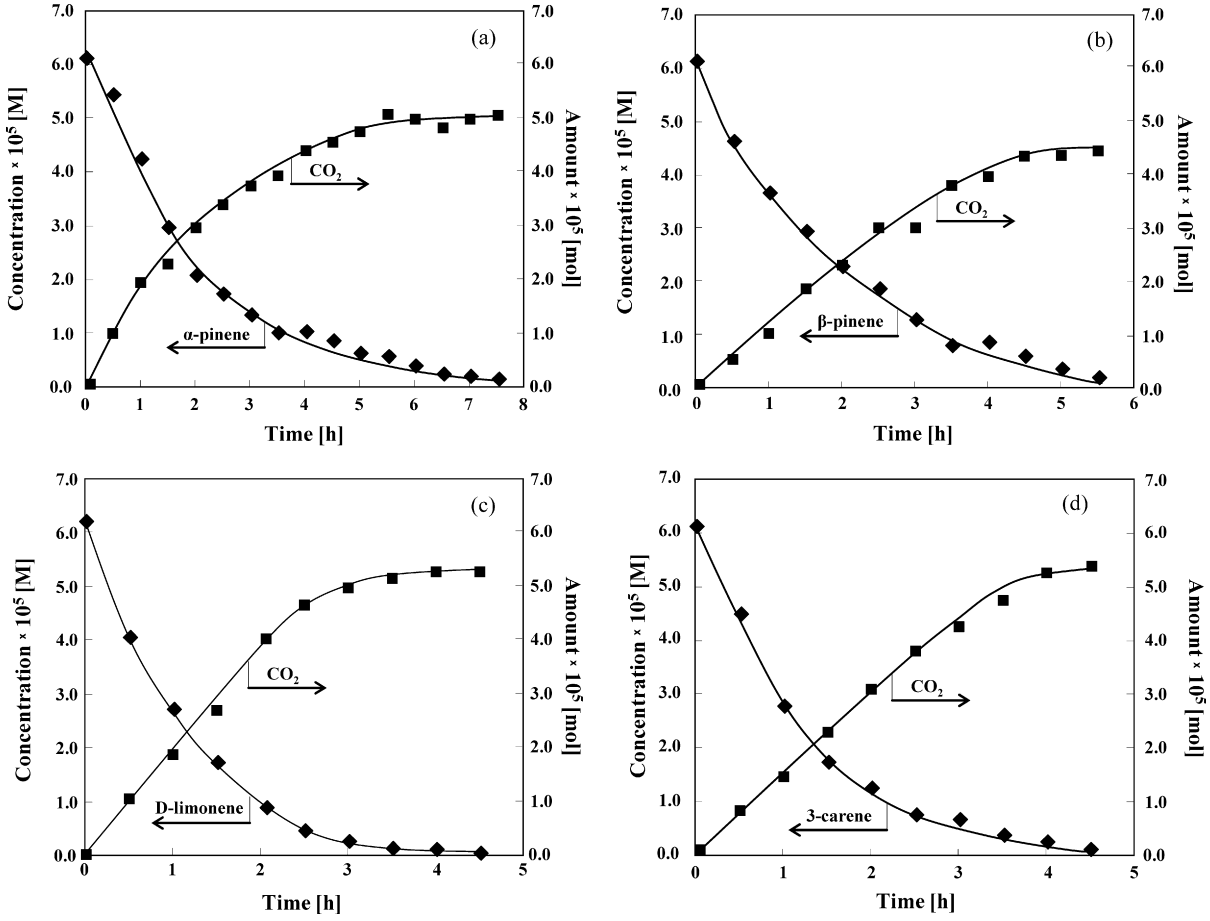

Fig. 4. Concentration of α-pinene (a); β-pinene (b); D-limonene (c); 3-carene (d) and CO_2 evolution versus irradiation time.

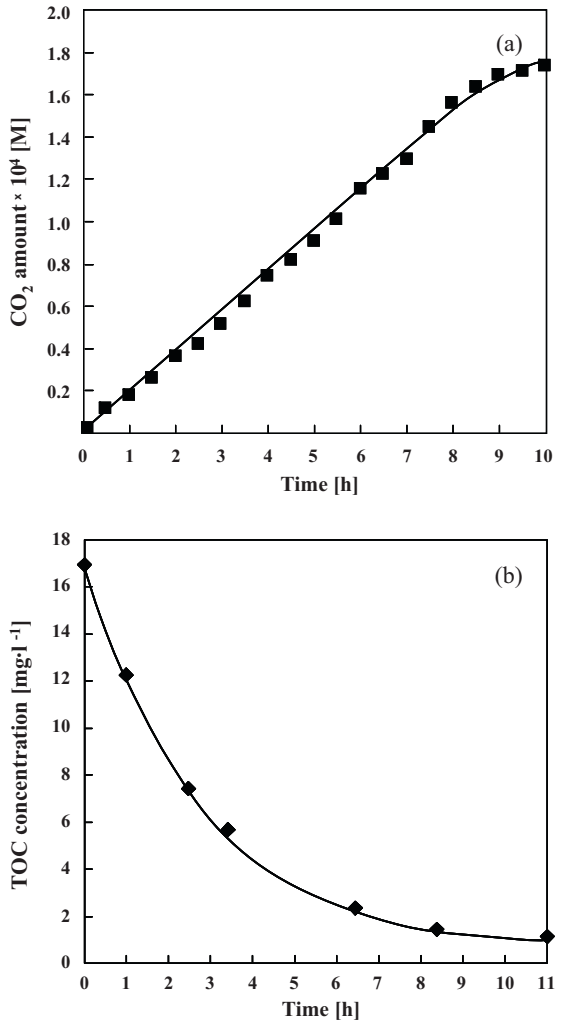

Fig. 5. (a) CO_2 evolution versus irradiation time for a run carried out by using a solution containing the four terpenes at a 2.47×10^{-4} M global concentration and (b) total organic carbon concentration versus irradiation time of sample B withdrawn in 2008.

3.4. Photocatalytic experiments

In order to study the adsorption of terpenes onto the surface of the photocatalyst, preliminary tests were carried out in the dark without irradiation. These tests indicated that terpenes scarcely adsorb on TiO_2. Moreover no reactivity was observed in runs performed under the same experimental conditions used for the photo-reactivity experiments but in the absence of catalyst, oxygen or light. It was concluded that the simultaneous presence of O_2, catalyst, and irradiation is needed for the occurrence of terpenes degradation process. Some results of the photocatalytic tests carried out on synthetic aqueous terpene solutions are shown in Fig. 4a–d where the concentrations of α-pinene, β-pinene, D-limonene and 3-carene and the amount of formed CO_2 versus irradiation time are reported. For all cases the substrate completely disappeared after irradiation times ranging between 4.5 and 8 h, depending on the terpene. The appearance of CO_2 was observed simultaneously with the disappearance of terpene. The amount of photo-produced CO_2 measured in the gas-phase was always lower than the stoichiometric amount estimated from the complete oxidation of the carbon contained in the starting substrate. However, the loss of carbon is of the same order of magnitude to that corresponding to the solubility of CO_2 in the reactant solution. This finding indicates that the substrates were completely mineralized during the photocatalytic runs. Some experiments were carried out with a solution containing the four terpenes at a 2.47×10^{-4} M global concentration corresponding to the sum of those used in the single component experiment, but only the photo-evolution of CO_2 was monitored (Fig. 5a). Indeed it was not the aim of this work to study in detail the mutual influence of the terpenes on the photodegradation rate, but only to test if the photocatalytic method could be in principle applied to a mixture of the studied molecules. The results showed that also in that circumstance the mineralization was complete after 10 h of irradiation, i.e. within obviously a longer time than when the single molecules were degraded. Finally a run was carried out by using the actual wastewater (sample B, 2008 untreated wastewater) of the citrus transformation factory. In Fig. 5b the total organic carbon concentration (TOC) versus irradiation time is reported. The disappearance in ca. 11 h of the organic carbon (the residual TOC was ca. 1 mg l^{-1}, i.e. in the experimental error of the instrument) indicated that also in an actual situation where probably other molecules are present, the photocatalytic technology can be applied.

4. Conclusions

The results presented in this paper indicate that V79 Hamster chinese cells survival decreased at about 50% after exposure to each of the four terpenes studied present in wastewater from a citrus transformation factory, but no additive or sinergistic effect was detectable when a mixture of terpenes was used. Moreover, each single terpene did not show any mutagenic significant effect, whereas the mixture of terpenes at the highest concentrations induced a mutagenic response that appeared to be synergistic in the HGPRT assay. The industrial biological treatment was quite effective, however residual concentrations of terpenes, still present in the treated samples, induced a slight increase of mutant clones. Photocatalytic tests carried out by using both synthetic and actual aqueous effluents in the presence of TiO_2 as the catalyst, indicated the possibility to photodegrade completely all the four terpenes, similarly to the biological treatment applied in the factory.

In conclusion, we have shown the potential hazard of the mixture of four components of citrus wastewater on environmental health and we advise that it is very important to evaluate the genotoxicity of the mixture of all compounds present in the citrus wastewater. The composition of citrus transformation plant wastewater is highly complex and relevant differences in the mutagenic response should be expected.

Acknowledgments

Authors thank the University of Palermo for financial support.

References

[1] E. Pichersky, J. Gershenzon, The formation and function of plant volatiles: perfumes for pollinator attraction and defense, Curr. Opin. Plant Biol. 5 (2002) 237–243.
[2] F. Loreto, P. Pinelli, F. Manes, H. Kollist, Impact of ozone on monoterpene emissions and evidence for an isoprene-like antioxidant action of monoterpenes emitted by Quercus ilex leaves, Tree Physiol. 24 (2004) 361–367.
[3] T.D. Sharkey, S. Yeh, Isoprene emission from plants, Ann. Rev. Plant Physiol. Plant Mol. Biol. 52 (2001) 407–436.
[4] H. Loza-Tavera, Monoterpenes in essential oils. Biosynthesis and properties, Adv. Exp. Med. Biol. 464 (1999) 49–62.
[5] R. Croteau, J. Gershenzon, Genetic control of monoterpene biosynthesis in mints (Mentha: lamiaceae). Genetic engineering of plant secondary metabolism, Rec. Adv. Phytochem. 28 (1994) 193–229.
[6] D. Tholl, Terpene synthases and the regulation, diversity and biological roles of terpene metabolism, Curr. Opin. Plant Biol. 9 (3) (2006) 297–304.
[7] A.E Edris, Pharmaceutical and therapeutic potentials of essential oils and their individual volatile constituents: a review, Phytother. Res. 21 (4) (2007) 308–323.

[8] J.A. Buhagiar, M.T. Camilleri Podesta, A.P. Wilon, M.J. Micallef, S. Ali, The induction of apoptosi in human meloma, brest and ovarian cancer cell lines using an essential oil extract from the conifer Tetraclinis articulate, Anticancer Res. 19 (6B) (1999) 5435–5443.
[9] J.R. Lazutka, J. Mierauskiene, G. Slapšyte, V. Dedonyte, Genotoxicity of dill (*Anethum graveolens* L.), peppermint (Mentha × piperita L.) and pine (*Pinus sylvestris* L.) essential oils in human lymphocytes and *Drosophila melanogaster*, Food Chem. Toxicol. 39 (5) (2001) 485–492.
[10] H.F. Mark, R. Naram, J.T. Singer, R.W. Rice, W.C. Bastan, L.J. Beauregard, P.H. Lamarche, Wood-drying condensate from Eastern white pine induced cytotoxicity and genotoxicity in vitro, Ann. Clin. Lab. Sci. 26 (1) (1996) 64–70.
[11] V.A. Isodrov, Non-methane hydrocarbons in atmosphere of boreal forest: composition, emission rates, estimation of regional emission and photocatalytic transformation, Ecol. Bull. 42 (1992) 71–76.
[12] C.H. Liu, A.K. Mishra, R.X. Tan, C. Tang, H. Yang, Y.F. Shen, Repellent and insecticidal activities of essential oils from *Artemisia princeps* and *Cinnamomum camphora* and their effect on seed germination of wheat and broad bean, Bioresour. Technol. 97 (15) (2006) 1969–1973.
[13] S. Garten, R.V. Falkner, Role of mentholated cigarettes in increased nicotine dependence and greater risk of tobacco-attributable disease, Prev. Med. 38 (6) (2004) 793–798.
[14] M. Schiavello (Ed.), Heterogeneous Photocatalysis, Wiley Series in Photoscience and Photoengineering, vol. 3, John Wiley and Sons, Chichester, 1997.
[15] G. Marcì, E. García-López, A. Di Paola, L. Palmisano, Photocatalytic oxidation mechanism of benzonitrile in aqueous suspensions of titanium dioxide, Catal. Today 129 (2007) 16–21, and references therein.
[16] L. Palmisano (Ed.), Processi e Metodologie per il trattamento delle Acque, Edizioni Spiegel, Milano, Italia, 2000.
[17] J. Pawliszyn (Ed.), Applications of Solid Phase Microextraction, Royal Society of Chemistry, Cambridge, UK, 1999.
[18] G. Vas, K. Vekey, Solid-phase microextraction: a powerful sample preparation tool prior to mass spectrometric analysis, J. Mass Spectrom. 39 (2004) 233–254.
[19] W. Cheng, M. Chou, C. Perng, F. Chu, Determining the equilibrium partitioning coefficients of volatile organic compounds at an air–water interface, Chemosphere 54 (7) (2004) 935–942.
[20] D. Zuba, A. Parczewski, M. Reichenbächer, Optimization of solid phase microextraction conditions for gas chromatographic determination of ethanol and other volatile compounds in blood, J. Chromatogr. B 773 (1) (2002) 75–82.
[21] S. Hamm, E. Lesellier, J. Bleton, A. Tchapla, Optimization of headspace solid phase microextraction for gas chromatography/mass spectrometry analysis of widely different volatily and polarity terpenoids in olibanum, J. Chromatogr. A 1018 (1) (2003) 73–83.